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Effects of a wild type strain and a mutant strain of the fungus *Verticillium lecanii* on *Meloidogyne incognita* populations in greenhouse studies

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Summary – A wild type strain of *Verticillium lecanii* and a UV-induced mutant were tested for ability to reduce populations of *Meloidogyne incognita* on tomato in the greenhouse. The two fungus strains were incorporated into a bran-alginate formulation and applied near the plant roots at three rates : 0.0009 % dry weight fungus/weight soil, 0.002 % w/w, and 0.02 % w/w. The mutant strain was also tested at 0.006 % w/w. Egg and juvenile numbers were counted and combined. Significant population decreases of 53 % and 66 % were observed in two of six experiments in which the mutant was applied at 0.002 %-0.006 % w/w. However, when results of all experiments were combined, neither the mutant nor the wild type strain significantly suppressed root knot nematode populations.

Résumé – ***Effets d'une souche sauvage et d'une souche mutante du champignon* Verticillium lecanii *sur les populations de* Meloidogyne incognita *élevées sur tomate en serre*** – La capacité d'une souche de *Verticillium lecanii* et celle d'un mutant induit par les UV à réduire des populations de *Meloidogyne incognita* sur des plants de tomates ont été testées. Les deux souches ont été incorporées à un mélange de son et d'alginate, et trois taux – 0,0009 %, 0,002 % et 0,02 % de poids sec de champignon par poids de sol – ont été placés à proximité des racines. La souche mutante a été également testée aux taux de 0,002 et 0,006 %. Le nombre d'œufs et de juvéniles a été déterminé. Des réductions significatives des populations, 53 % et 66 %, ont été observées dans deux des six expériences où la souche mutante avait été appliquée aux taux de 0,002 % et 0,006 % de poids sec par poids de sol. Cependant, si les résultats de toutes les expériences sont combinés, ni la souche sauvage, ni la souche mutante ne réduisent de façon significative les populations du nématode.

Key-words : *Verticillium,* nematode-parasitizing fungi, biological control, mutant, *Meloidogyne,* nematodes.

Verticillium lecanii (A. Zimmermann) Viégas is antagonistic to a number of organisms, including insects, fungi, and nematodes (Hussey, 1984; Harper & Huang, 1986; Uma & Taylor, 1987; Rodríguez-Kábana & Morgan-Jones, 1988; Heintz & Blaich, 1990). This species was shown to penetrate the cyst wall of *Heterodera schachtii,* colonizing the cyst cavity following penetration (Hänssler & Hermanns, 1981). The fungus also colonized eggs and juveniles of *H. schachtii* and secreted enzymes that degraded cyst wall and egg constituents (Hänssler, 1990). *Verticillium lecanii* was isolated from cysts and females of *Heterodera glycines* Ichinohe in field soil (Gintis *et al.,* 1983), and a strain of *V. lecanii* significantly decreased viability of soybean cyst nematode eggs in a Petri dish assay (Meyer *et al.,* 1990). The latter strain was selected for further study as a possible biocontrol agent for plant-parasitic nematodes, both because the fungus has potential to act against nematodes, and because successful production of *V. lecanii* for insect control demonstrates that it is suitable for commercial applications.

As part of this research, the strain was shown to have some tolerance to the fungicide benomyl (Meyer *et al.,* 1991), and mutants with increased benomyl tolerance were induced with ultraviolet radiation (Meyer, 1992). Reasons for induction of these mutants included the following : increased potential to survive in fields where benomyl is applied, ability to incorporate the strains into a delivery system where benomyl has been added to reduce growth of other fungi, use of resistance or increased benomyl tolerance to aid in strain identification, and possible enhanced biocontrol potential as a result of the genetic manipulation. The latter has been observed in other fungus species (Abd-El Moity *et al.,* 1982; Papavizas *et al.,* 1982; Papavizas & Lewis, 1983; Baker, 1989), but little research has been published on induction of fungus mutants for nematode control. A study was conducted comparing a wild type strain of *Paecilo-*

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myces lilacinus (Thom) Samson with benomyl-resistant biotypes for control of *Meloidogyne incognita* on tomato (Gaspard & Mankau, 1985; Gaspard 1986). In those experiments, nematode egg numbers were not significantly reduced by the fungus strains.

The purpose of the current investigation was to test a wild type strain of *V. lecanii* and an induced mutant for ability to affect populations of *M. incognita* on tomato. The objectives of this study were *i)* to ascertain whether either strain decreased root knot numbers in the soil, *ii)* to compare the effects of the mutant and the wild type strains, and *iii)* to determine whether application levels affected results.

Materials and methods

Fungus inoculum

Verticillium lecanii was obtained from the American Type Culture Collection (number 58909). Mutant strain M2S1 (Agricultural Research Service Culture Collection, NRRL 18726) was induced from strain 58909 with ultraviolet radiation (Meyer, 1992). To produce inoculum for greenhouse experiments, fungi were grown on potato dextrose agar (PDA) in Petri dishes for one week. These colonies were cut into pieces with a scalpel and homogenized in potato dextrose broth (PDB) in a Waring Blendor; pieces from one to five Petri dishes were combined, with an estimated 40 ml broth used per Petri dish of fungus. The fungus/broth suspension was poured into 1 liter Erlenmeyer flasks. Each flask was inoculated with fungus/broth suspension containing hyphae and conidia approximately equivalent in mass to one Petri dish colony. The final broth volume per flask was 250 ml. Flasks containing PDB and *Verticillium* were rotated on orbital shakers (240 rpm) for 2 days at 25 °C. Fungi were harvested by centrifugation of the broth cultures at 9000 rpm for 10 min (13 000 *g*) in a Sorvall® GSA rotor and collection of the fungus pellet. Wet mycelium and conidia were stirred in an equal volume of water and homogenized to a slurry in a VirTis mechanical homogenizer. The slurry was then incorporated into alginate prills (Fravel *et al.*, 1985) for application to the soil. One hundred g fungus (wet weight) and 5 g wheat bran (ground to particle size of less than 1 mm) were added per liter of alginate slurry. Fungus wet weight to dry weight ratios were determined by weighing extra fungus that had been dried overnight at 50 °C.

Nematode inoculum

Meloidogyne incognita eggs were extracted from greenhouse stock cultures (procedure modified from Hussey & Barker, 1973; McClure *et al.*, 1973; Taylor & Sasser, 1978). Infected tomato roots were placed into a 0.525 % sodium hypochlorite solution for 45 s to release the eggs. The roots and sodium hypochlorite solution were then poured over nested 60 mesh (pore size 250 µm) and 500 mesh (pore size 25 µm) screens and rinsed thoroughly. The eggs were collected off the 500 mesh screen, rinsed into centrifuge tubes, and centrifuged at 1 700 rpm (450 *g*) for 3 min. The supernatant was poured off, a 1 M sucrose solution was added to the tubes, the suspension was centrifuged again at the same time and speed, and the supernatant was collected on a 500 mesh screen. The eggs were rinsed thoroughly and collected in 100 ml water. A Peter's counting slide was used to obtain the inoculum rate of 5000 eggs per pot.

Greenhouse Experiments

Tomato (*Lycopersicon esculentum* Mill.) cv. Marglobe seeds were germinated in Terra-Lite® Redi-Earth Peat-Lite mix (Grace Sierra, Horticultural Products Co., Milpitas, CA, 95035-2003). Seedlings were germinated under supplemental lighting (400 watt high pressure sodium bulbs, 16 continuous hours per day) during September-April. Supplemental lighting was not used May-August. Three weeks after sowing, the tomato plants were transplanted into pots filled with unsteamed soil mix (*ca.* 77 % sand, 16 % silt, 7 % clay, pH 6.7-7.2) made from three parts compost (16 m^3 top soil : 8.4 m^3 manure : 36 kg 5/10/5 : 109 kg high magnesium dolomitic lime) and 1 part sand. Experimental units were 20-cm-diameter pots for the mutant/wild type comparison experiments, and 15-cm-diameter pots for the mutant only experiments and the autoclaved prill experiments. After two plants were transplanted into each pot, the pots were moved to natural lighting. Three weeks later, four holes were made in the soil near the plant roots. Each pot was inoculated with 4 ml of aqueous root knot egg suspension containing approximately 5000 nematodes eggs (1 ml suspension containing 1250 eggs was added to each hole). Prills were added to the same holes in all but control pots. Control pots did not receive prills. Pots were arranged in a randomized complete block design. Plants then grew for 9 weeks in summer conditions or 11 weeks in winter before eggs were collected and counted. Greenhouse temperatures were maintained as close as possible to 27 °C (16-43 °C range). Sierra™ Poinsettia Mix was applied as a top dressing (10 g per 15-cm pot; 20 g per 20-cm pot) every 1.5-2 months or when yellowing was observed.

At the conclusion of each experiment, the tomato plants were cut at the soil surface and the upper portions were discarded. The pot contents (including soil and tomato roots) were placed into a bucket of tap water, and the soil was gently cleaned off the roots (leaving the egg masses still attached to the roots). Nematode eggs and juveniles were collected from the roots in each pot in a manner similar to that previously described under " nematode inoculum ". One egg/juvenile sample was prepared per pot. Three one-ml aliquants of egg/juvenile suspension were taken from each sample, and the mean number of healthy and diseased eggs (excluding

empty eggs) and juveniles per pot was estimated from these counts. The number of juveniles counted was so small that counts are referred to in this paper as numbers of eggs.

In the 20-cm diameter pots, three prill application rates were tested for each fungus. Four pots were used for each application rate and for controls in each repetition, and the study was conducted three times (Experiments 1-3; n = 12 for each treatment). The application rates were 0.15, 0.5, and 5.0 g prills/pot (0.003 %, 0.01 %, and 0.1 % weight prills/weight soil), approximately equivalent to 0.05, 0.1-0.15, and 1.0-1.5 g dry fungus per pot (0.0009 %, 0.002 %, and 0.02 % w/w). The mutant fungus was then tested further at one application rate : 0.5 g prills/pot (*ca* 0.12 g dry fungus/pot). In 15-cm-diameter pots, this was approximately 0.025 % prills/soil (w/w), and 0.006 % dry weight fungus/weight soil. The experiment was conducted three times (Experiments 4-6), with fourteen control pots and fourteen fungus-treated pots per experiment (n = 42 per treatment).

Prills containing mutant *V. lecanii* were autoclaved so that the fungus was no longer viable, and tested for effects on numbers of nematode eggs (Experiments 7 and 8). Four control pots and four pots with autoclaved prills were used in each experiment (n = 8 per treatment). Prills were applied at the highest rate : 5.0 g per 15-cm-diameter pots.

Retrieval of fungi from soil

As Experiments 1-6 were concluded, soil from each pot (including control pots) was plated onto agar to check for the presence of *Verticillium*. To isolate *V. lecanii*, 10 g of soil per pot were stirred in 50 ml water (dilution 1), and 1 ml of the suspension was then added to 9 ml water (dilution 2). For Experiments 1-5, approximately 0.05 ml of each dilution was plated onto each of two or three plates of the following agars : Aushers medium No. 2 (Ausher *et al.*, 1975), or PDA amended with benomyl (Benlate® 50 Wettable Powder or DF, E. I. du Pont de Nemours & Co., Wilmington, DE, 19801) and antibiotics (PDA ABE 100). PDA ABE 100 was made with 39 g PDA, 970 ml distilled water, 0.2 g benlate in 20 ml distilled water, and 0.3 g streptomycin sulfate plus 0.3 g tetracycline in 10 ml sterile water. The flask in which the antibiotics were mixed in water was rinsed with 6 ml EtOH, which was added to the medium. For Experiment 6, only PDA ABE 100 was used.

Analysis of data

Individual experiments were analyzed for significance. Experiments 1-3 were combined for analysis. Each fungus application rate was compared with untreated controls, and all rates for each fungus were combined for comparison between fungi and comparison with untreated controls. Untreated controls were compared with mutant fungus applied at 0.5 g prills per pot in Experiments 4-6, and in Experiments 1-6 combined. Autoclaved fungus prill treatments were compared with untreated controls (Experiments 7 and 8). Differences among wild type treatments, mutant treatments, and affect of rate were tested using single degree of freedom contrasts ($P < 0.05$). Planned comparisons were used to determine whether differences between autoclaved fungus prill treatments and untreated controls were significant ($P < 0.05$). Variances were checked for homogeneity, and data transformation was not needed. PROC GLM from SAS was used (SAS Institute Inc., Cary, NC; SAS/STAT™ User's Guide, Release 6.03 Edition, 1988 and SAS/GRAPH® Software : Usage, Version 6, First Edition, 1991).

Results

When results of Experiments 1-3 were combined, no significant decrease in number of nematode eggs was found with any application rate for either fungus strain (Fig. 1). A significant reduction (66 %) in nematode eggs occurred in Experiment 2 with the 0.5 g prills/pot mutant fungus treatment, but a 67 % increase ($P = 0.052$) in egg numbers was recorded (Experiment 3) at the same application rate of mutant fungus. Application of the wild type strain at 0.15 g prills/pot

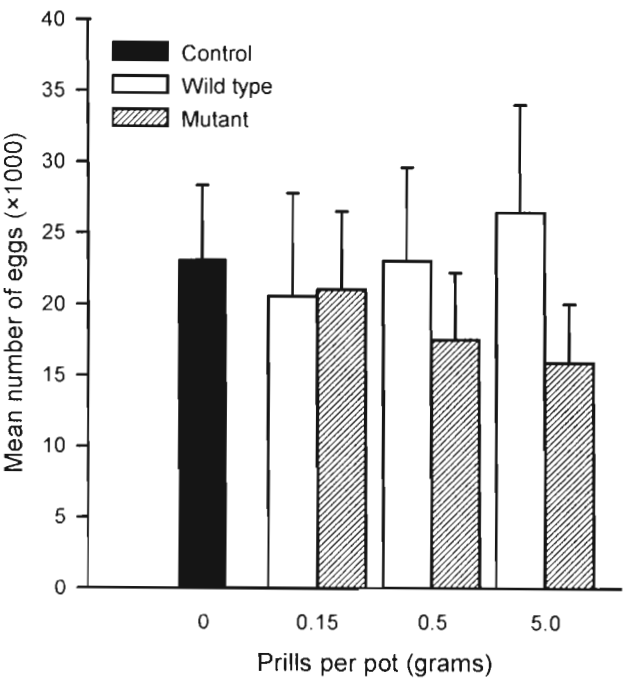

Fig. 1. *Effect of wild type and mutant strains of* Verticillium lecanii *on number of root knot nematode eggs produced in greenhouse pots. Control pots were not treated with prills. Host : tomato. Results are from Experiments 1-3 combined, with 4 pots per treatment in each experiment. The three application rates tested were 0.15, 0.5, and 5.0 g prills per 20-cm-diameter pot.*

caused a 70 % reduction in Experiment 1, but the F value was not significant. Overall, an application rate of 0.15 g prills of wild type fungus/pot resulted in an 11 % decrease in nematode populations when compared with controls (Fig. 1). A rate of 0.5 g prills/pot resulted in only a 0.2 % decrease compared to controls. An application rate of 5.0 g prills/pot resulted in the largest nematode egg counts; there was a 14 % increase compared to the controls, but the difference was not significant. Application of the mutant fungus resulted in a trend where egg population levels decreased with increased amount of added fungus. At 0.15 g prills/pot, the population levels estimated after treatment with mutant fungus were similar to those found with the wild type strain (9 % decrease compared to controls). A mean 24 % reduction compared to controls occurred at an application rate of 0.5 g prills/pot, and a 31 % reduction compared to controls was found at an application rate of 5.0 g prills/pot.

When Experiments 1-3 were combined, significant differences were not found between wild type fungus treatments and mutant fungus treatments. However, when all rates of application were combined for Experiments 2 and 3, application of the wild type strain resulted in significantly more nematode eggs per pot than application of the mutant strain.

Since, on average, application of mutant fungus resulted in the greatest decrease in egg numbers, the mutant alone was selected for additional tests (Experiments 4-6) with increased numbers of replicates per experiment. A statistically significant reduction (53 %) in egg numbers occurred with application of the mutant fungus in Experiment 4, but results from Experiments 5 and 6 were not significant. When Experiments 4-6 were combined for analysis, there was no significant decrease in egg numbers. When the results with the 0.5 g prills/pot mutant application rate from Experiments 1-6 were combined, the egg numbers after fungus application were 36 % lower than population levels in the controls, but the difference was not significant (Means ± SD, n = 54; Untreated control = 118 065 ± 209 312; mutant fungus treatment = 75 748 ± 112 772).

No significant difference in egg numbers was found between the eight pots treated with autoclaved fungus prills (113 891 ± 87 434) and the eight pots without prills (98 500 ± 68 549).

Samples were taken from all 168 pots in Experiments 1-6 to determine whether *V. lecanii* could be recovered from the soil. The mutant strain had been inoculated into 78 pots, the wild type into 36 pots, and 54 pots contained controls with no fungus. In Experiment 1, each fungus strain was isolated from a pot into which it had been inoculated. The wild type strain was recovered on one Petri dish of Ausher's medium, soil dilution 2, from a pot originally inoculated with 5.0 g prills. The mutant was recovered on two Petri dishes of PDA ABE 100, soil dilution 2, from one pot originally inoculated with 5.0 g prills. The fungi were not recovered from any other soil samples.

Discussion

In a preliminary Petri dish assay (Meyer, unpubl.), mutant strain M2S1 of *V. lecanii* significantly decreased viability of *M. incognita* eggs, while the wild type did not. However, live and dead uncolonized eggs can be difficult to distinguish from each other, and even colonization does not prove parasitism, since colonization may occur after egg death. Consequently, tests in the soil are essential for determining whether an organism exhibits promise as a biocontrol agent. The greenhouse studies reported here were conducted as the next step in examining ability of the fungus strains to affect root knot nematode populations. When the greenhouse experiments were combined for analysis, no application rate of either fungus strain caused a statistically significant decrease in root knot nematode populations. In the analyses of individual experiments, statistically significant reductions were recorded in two of six trials where the mutant strain was applied at 0.002 %-0.006 % weight dry fungus/weight soil. However, the most substantial increase in egg numbers was also recorded when the mutant fungus was applied at 0.002 % w/w (Experiment 3). At all but the lowest application rate, the egg numbers counted after application of the mutant strain tended to be lower than numbers recorded after wild type application, even though benomyl was not applied to the plants. However, differences between the strains were significant only when Experiments 2 and 3 were combined without Experiment 1.

Alginate granules have been successfully utilized in research studies on management of plant-parasitic nematodes (Cabanillas *et al.*, 1989; Schuster & Sikora, 1992). Incorporation of *V. lecanii* into alginate prills resulted in production of granules containing viable fungus, as indicated by fungus growth from prills plated onto agar, and by isolation of *V. lecanii* from steamed soil that had been treated with prills (Meyer, unpubl.). However, recovery of *V. lecanii* from the unsteamed soil used in the *M. incognita* experiments was negligible, even with the use of semi-selective media. It is not known whether the fungus could not be recovered in most cases because of difficulty in developing a semi-selective medium, or because the fungus did not compete well in the unsteamed soil. Quantitative assays for these strains of *V. lecanii* in soil have been difficult to develop, but would contribute significantly to biocontrol work.

Application of alginate granules without fungus caused a reduction in numbers of new cysts formed by *Globodera pallida* (see : Schuster & Sikora, 1992). In the current study, no effect on *M. incognita* was recorded when prills without viable *V. lecanii* were applied to soil.

Application rates of 1 % w/w or higher (about 22 700 kg/ha) are not uncommonly studied when fungi are tested for biological control of nematodes. Such studies do indicate that a fungus can affect the nematode population, but not at rates that can realistically be applied to fields. The prills were added to the current experiments at rates approximately equal to 70 kg/ha, 200-550 kg/ha, and 2200 kg/ha, and were applied near the roots where the fungus could best protect the plants from nematodes. While nematode populations were significantly reduced in two experiments when prills containing mutant fungus were added at the 200-550 kg/ha rate, no statistically significant population decreases were recorded when the mutant strain was applied at the highest application rate. These studies may indicate that large amounts of fungus are not necessarily more efficacious for reducing nematode populations. However, the result must be interpreted with caution in this case because of the inconsistency in the effects of the mutant at the 220-550 kg/ha application rate.

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